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(54) **Application of phospholipases in animal feed**

Verwendung von Phospholipasen in Tierfutter

Utilisation de phospholipases dans l'alimentation animale

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(56) References cited:  
**EP-A- 0 359 425** **EP-A- 0 426 211**  
**EP-A- 0 449 375** **EP-A- 0 619 079**  
**GB-A- 2 267 033**

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**Description****Field of the invention**

5 [0001] The present invention relates to the application of enzymes in feed for livestock.

**Background of the invention**

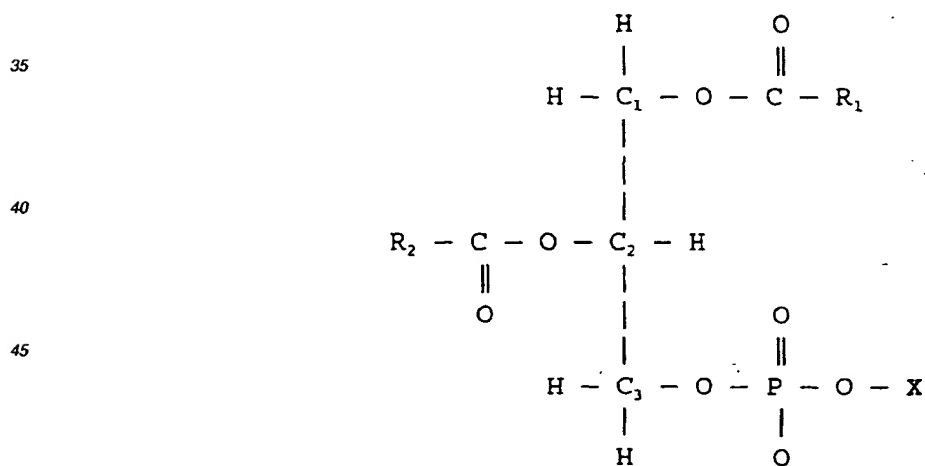
10 [0002] A number of enzymes are secreted in the gastrointestinal tract of animals to digest food. Each of these enzymes acts on specific components in a specific environment of a part of the gastrointestinal tract. Pepsins, for example, are active in the acidic environment of the stomach, whereas other proteases such as chymotrypsin and carboxypeptidases show activity in the upper part of the small intestine at pH 6-7. Many such enzymes need a precursor before being activated. Pepsin, for example, is only formed from pepsinogen in an acidic environment. Chymotrypsin and carboxypeptidases are both secreted in an inactive form and are activated by the protease trypsin.

15 [0003] The digestion of fat is a complex process. Most fat in diets for animals is available in the form of triglycerides. These triglycerides are hardly if at all absorbable by the intestine and need to be degraded to mono- and diglycerides, glycerol and free fatty acids. This conversion is catalyzed by the enzyme lipase which is secreted by the pancreas. This enzyme is active on the interface of water and oil. For good digestion it is essential to have very small droplets of fat in an oil-in-water emulsion. Emulsifiers are surface-active substances that allow the dispersion of fat in a water phase. The most important emulsifier in the gastrointestinal tract is bile. Bile is secreted by the liver and may be stored in the gall bladder. Bile contains a.o. bile acids and salts, cholesterol and phospholipids. Small particles, micelles, are formed by the mixture of the bile components with the (remaining) triglyceride products.

20 [0004] These micelles are diffused to the jejunal epithelial cells, where their contents are released and absorbed. In these epithelial cells, triglycerides are reconstituted. Together with cholesterol, cholesterol esters, phospholipids and proteins, they form new, water soluble, particles termed chylomicrons.

25 [0005] Phospholipids such as lecithin are enzymatically degraded by action of phospholipases A and B which are also secreted by the pancreas.

30 [0006] Lecithin is a mixture of both polar and neutral lipids in which the content of polar lipids is at least 60%. Because of their hydrophobic/hydrophilic character polar lipids (and thus lecithins) are used as emulsifiers. Polar lipids include (glycero)phospholipids and glycolipids. The basic structure of a glycerophospholipid is as follows:



X = Choline Ethanolamine Inositol Serine Hydrogen

55 [0007] Glycerophospholipids basically consist of a glycerol moiety with fatty acids at the C1- and C2 position. The C3-position is esterified with phosphoric acid. This phosphoric acid is often linked with an alcohol group thus generating the following compounds:

- phosphatidyl-ethanolamine (PE; X = ethanolamine)
- phosphatidyl-choline (PC; X choline)

- phosphatidyl-serine (PS; X = serine)
- phosphatidyl-inositol (PI; X = inositol)
- phosphatidyl acid (PA; X = hydrogen)

5 [0008] Glycerophospholipids bearing just one (instead of the usual two) fatty acid residues are called lyso-phospholipids.

[0009] Lecithin is used as an emulsifier in numerous applications including food and feed. Emulsifiers are surface-active substances that allow the dispersion of an oil liquid phase in a water phase. Emulsifiers possess both hydrophilic and lipophilic groups within the same molecule. The ratio of hydrophilic to lipophilic groups, known as the HLB value, is a characteristic indicator for emulsifiers.

10 [0010] Fat-soluble hydrophobic emulsifiers have HLB values in the range of 0 to less than 10 while water-soluble compounds have HLB values between above 10 and 20.

[0011] Emulsifiers such as lecithin are added to animal feed to achieve an improved nutritive value of the feed or to achieve a better dispersion in the case of liquid feed. It is also known to add lysolecithin to animal feed (under the trade name Lysoforte® sold by the Kemin company) which has improved emulsifying properties leading to a better nutritive value (Pluimveehouderij 24: 20-21 (March 18, 1994).

15 [0012] The emulsifying properties of lecithin are not only exploited in livestock production by inclusion of lecithin in dry rations but also in areas where animals are given liquid feed containing a large proportion of fat. These are primarily milk replacements for calves and sow milk substitutes for piglets. The function of lecithins is to produce the finest possible dispersion of the fat in the ready prepared liquid feed. The fine dispersion results in improved digestibility of the fat by the animals. In addition, the lecithin exhibits a favourable effect on the settling of insoluble constituents in a liquid feed.

20 [0013] In recent years, the feed industry has started to use industrially produced enzymes to complement enzymes produced in the gastrointestinal tract of the animals. Examples comprise phytases,  $\alpha$ -amylases, proteases, and various plant cell wall degrading enzymes. However, nowhere in the prior art has the direct addition of phospholipases to animal feed for the purpose of promoting the growth of animals been described since the animals themselves already secrete large amounts of these enzymes in the upper part of the small intestine.

25 [0014] EP-A-0 619 079 discloses the use of *inter alia* phospholipids as coatings for granulates containing biologically active substances to be include in feed for ruminants. The coating serves to protect the biologically active substances in the rumen, in order to allow subsequent digestion and absorption thereof via the post-abomasum digestive organs. EP-A 0 619 079 further discloses that optionally a phospholipase can also be incorporated in the protective coatings in order to aid in their hydrolysis, however, EP-A 0 619 079 does not disclose or suggest that phospholipases can be added to feed in order to promote growth or to improve the efficiency of feed utilization

30 [0015] GB-A-2 267 033 is concerned with promoting growth, however, GB-A-2 267 033 teaches to add a kit comprising the phospholipid lecithin together with a *Streptomyces* strain to silage. It was suggested that the *Streptomyces* strain is capable of producing a phospholipase A2 during fermentation of the silage. It follows that the use of said kit is limited to animal feed of which the production process comprises a fermentation stage which is compatible with phospholipase production by said *Streptomyces* strain. Hence, there is still a need for a widely applicable, versatile and ready-for-use phospholipase feed-additive for improvement of the efficiency of feed utilization and/or for the promotion of growth of animals.

### Summary of the invention

45 [0016] The present invention provides a process for improving the efficiency of feed utilization in which an animal is fed a diet which comprises a composition comprising feed substance and a ready for use phospholipase additive.

[0017] The present invention also provides a process for promoting the growth of animals in which an animal is fed a diet which comprises a composition comprising feed substance and a ready for use phospholipase additive.

[0018] The invention further provides animal feed compositions comprising feed substance and a ready for use phospholipase additive.

50 [0019] In addition the invention provides a process for the production of an animal feed which comprises mixing a ready for use phospholipase with feed substance.

### Detailed description of the invention

55 [0020] The present invention discloses the use of exogenously added and ready for use phospholipases in feed for animals to improve the emulsifying properties of phospholipids in the gastrointestinal tract and thereby improving the efficiency of feed utilization and/or promoting the growth of the animal. Promoting the growth of animals is herein defined as promoting growth in terms of weight gain in time (growth rate) and/or promoting growth in terms of feed

efficiency (feed conversion ratio).

[0021] Phospholipases which may be used in the invention include: phospholipase A1 (EC 3.1.1.32), phospholipase A2, phospholipase B (lysophospholipase), phospholipase C and phospholipase D.

5 [0022] Specifically, the present invention discloses the application of feed to which phospholipase A2 (EC 3.1.1.4) is added. Phospholipase A2 may be produced by isolation from e.g. porcine pancreas as a byproduct from e.g. insulin production. Alternatively, phospholipase A2 may be produced recombinantly by expression of a heterologous gene in a micro-organism such as e.g. *Kluyveromyces lactis*. The enzyme is obtained from such microorganisms by means of fermentation and down-stream processing to recover the enzyme.

10 [0023] Another possibility for the exogenous addition of phospholipases to lecithin containing animal feed, is to add phospholipase containing transgenic plant materials, preferably transgenic seed, in which the phospholipase has been synthesized through heterologous gene expression. To obtain this the gene encoding the phospholipase is cloned in a plant expression vector, under control of the appropriate plant expression signals, e.g. a tissue specific promoter, such as a seed specific promoter. The expression vector containing the phospholipase gene is subsequently transformed into plant cells and transformed plant cells are selected for regeneration into whole plants. The thus obtained  
15 transgenic plants can be grown and harvested, and those parts of the plants containing the heterologous phospholipase, can be included in animal feed, either as such or after further processing. The heterologous phospholipase may be contained in the seed of the transgenic plants or it may be contained in other plant materials such as roots, stems, leaves, wood, flowers, bark, and/or fruit.

20 [0024] Thus a phospholipase additive is understood to mean a phospholipase which is not a natural constituent of the main feed substances or is not present at its natural concentration therein, e.g. the phospholipase is added to the feed separately from the feed substances, alone or in combination with other feed additives, or the phospholipase is an integral part of one of the feed substances but has been produced therein by recombinant DNA technology.

25 [0025] A ready for use phospholipase additive is herein defined as an additive that is not produced *in situ* in animal feed. A ready for use phospholipase additive may be fed to animals directly or, preferably, directly after mixing with other feed constituents. Ready for use phospholipase additives do include phospholipases that are in the inactive form but that can be activated in the GI-tract, e.g. by proteolytic processing.

30 [0026] A preferred feed contains phospholipid, preferably lecithin as present in raw materials as for example either full fat soybeans, full fat rapeseed, soybean oil, rapeseed oil or any other oilseed or oil rich in lecithin in addition to the exogenously added phospholipase, which is preferably (microbially produced) porcine phospholipase A2. However, the feed does not need to comprise phospholipid as the pancreas already secretes phospholipid.

[0027] It is another aspect of this invention that phospholipase, preferably (microbially produced) porcine phospholipase A2, is included in milk replacers containing lecithin for young animals. This improves the digestibility of fat by young animals.

35 [0028] It is yet another aspect of this invention that a phospholipase is included in diets of fish and crustaceans in order to improve growth and feed conversion ratio.

[0029] Upon treatment with e.g. phospholipase A2 (PLA2), the HLB value of lecithin is raised from 7 to approximately 8 or 9 which may contribute to the beneficial effects of phospholipase A2 treatment on emulsification properties of lecithin.

40 [0030] Porcine phospholipase A2 exhibits no activity *in vitro* below pH 6.0. The prevailing pH in the GI-tract of monogastric animals is below 6.0 in the crop and stomach.

[0031] One would not expect any beneficial effect of the addition of phospholipase A2 since:

- 45 a) no activity is expected of an added phospholipase in the crop and stomach as a consequence of a mismatch between prevailing pH and pH dependency of the enzyme; and  
b) the animals themselves secrete large amounts of this enzyme in the upper part of the small intestine where the prevailing pH is in line with the pH dependency of the enzyme.

[0032] Surprisingly, it was found that the addition of exogenous porcine phospholipase A2 results in a markedly improved feed conversion ratio in broilers (Example 3).

50 [0033] Apart from monogastric animals, phospholipase A2 may also be used advantageously in polygastrics. During early lactation of high producing dairy cows for example, it is of interest to include high levels of fat in their diets to compensate in part for the large negative energy balance. From literature it is known that the digestibility of fatty acids in the GI-tract of dairy cows varies as a function of *inter alia* ration composition and source of fat. Digestibilities of fatty acids have been found to vary between 87% for a diet containing 500 g of saturated fat high in palmitic acid (C16:0) to 64% for a diet containing 1000 g of saturated fat high in stearic acid (C18:0) (Weisbjerg et al., Acta Agric. Scand. Section A, Animal Sciences 42 p. 115-120, 1992).  
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[0034] A large part of the variation in digestibility of fatty acids in dairy cows is explained by variations in digestibility in the small intestine (Ibid, p.114-120). Action of phospholipase A2 in the small intestine of dairy cows is likely to

enhance the digestibility of fatty acids.

[0035] Proteins such as the enzyme phospholipase A2 are usually degraded rapidly in the rumen. As a consequence, these proteins should be delivered to the small intestine in a way that prevents degradation in the rumen. A number of formulation methods are available to persons skilled in the art to protect enzymes from inactivation in the rumen.

[0036] A significant improvement has also been found in fat digestibility by non-ruminating calves upon addition of phospholipase, specifically porcine phospholipase A2 (Example 4).

[0037] Diets for fish and crustaceans are also often supplemented with relatively high concentrations of phospholipids in order to achieve an acceptable growth, health and feed conversion. In accordance with the invention phospholipase may also be added to these diets to further improve growth and feed conversion ratio.

[0038] It is yet a further aspect of the invention that the addition of phospholipase, and optionally phospholipid, to animal feed allows to reduce the amounts of expensive feed ingredients, such as vitamins and/or colorants, to be incorporated in the feed.

[0039] Phospholipase may be added to feed in a concentration which varies as a function of type and concentration of phospholipid and target animal generally between about 1,000 - 5,000,000 international units (IU, for definition see Example 1) per kg of phospholipid. Preferably about 10,000 - 500,000 IU are added per kg of phospholipid. In general, animal feeds contain about 1-2 g of phospholipid per kg. Consequently, a range of 1 - 10,000 IU/kg, or preferably 10 - 1,000 IU/kg of feed will be appropriate, however, most preferred is a range of about 100 - 1,000 IU/kg feed. It follows that the dosage of phospholipase added to the feed can be adjusted in case of an unusual phospholipid content of the feed.

[0040] In a preferred embodiment of the invention the phospholipase is obtained using recombinant DNA technology. The phospholipase is recombinantly produced by heterologous expression of a phospholipase gene or cDNA in a suitable host organism or, alternatively, by homologous overexpression of a suitable endogenous gene.

[0041] The specific embodiments of the present invention described herein use porcine phospholipase A2 as produced by heterologous gene expression in the yeast *Kluyveromyces lactis*. However, the skilled person will understand that phospholipases obtained from other sources will also function in the present invention. Such phospholipases may be derived from other mammals such as e.g. rats, mice or humans, for all of which the phospholipase A2 genes are available in the art. Alternatively, the phospholipases may be derived from organisms other than mammals, such as e.g. microbial or even plant phospholipases.

[0042] Similarly, the microorganism used for the production of the phospholipase used in the invention is not necessarily limited to the yeast *Kluyveromyces lactis* only. Apart from *K.lactis*, successful heterologous expression of the porcine phospholipase A2 gene has been reported in *Escherichia coli*, *Saccharomyces cerevisiae* and *Aspergillus niger* (reviewed by Swinkels *et al.* 1993, Antonie van Leeuwenhoek 64, 187-201). It is therefore expected that successful (heterologous) expression of the phospholipases of the invention can be obtained in a wide range of microorganisms. Preferred microorganisms for this purpose are bacteria of the genera *Bacillus* and *Escherichia*, yeasts of the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, *Yarrowia*, *Candida*, or filamentous fungi of the genera *Aspergillus*, *Fusarium*, and *Trichoderma*.

[0043] Regarding the methods for expression of phospholipases in transgenic plant materials, preferably in seed, we refer to International Application WO 91/14772, which discloses general methods for the (heterologous) expression of enzymes in plants, including methods for seed-specific expression of enzymes.

[0044] The skilled person will understand that addition of the phospholipase in the form transgenic plant material, e.g. transgenic seed containing the phospholipase, may require the processing of the plant material so as to make available the enzyme, or at least improve its availability. Such processing techniques may include various milling and grinding techniques or thermomechanical treatments such as extrusion or expansion.

[0045] The present invention is not limited to the use of *Streptomyces* strains capable of producing a phospholipase A2 during fermentation of silage, which *inter alia* provides the following advantages:

- Any phospholipase can be included. This allows to use phospholipases which are endogenous to the animal in which the enzyme is to be applied, which will facilitate obtaining product-approval from the regulatory authorities
- This further allows to select a phospholipase most suited for application as feed additive.
- The need to ferment the feed in order to produce the enzyme *in situ* is obviated. This allows to control precisely the amount of phospholipase additive in the feed, which is important in view of the optimum in the range phospholipase concentrations in certain applications (see Example 3).
- It also allows great flexibility in the formulation of both the enzyme additive and the feed comprising the same.
- The addition of mammalian phospholipase A2 provides an unexpectedly high effect on growth promotion.

**Example 1****Expression of porcine Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in the yeast *Kluyveromyces lactis***

5 [0046] The identification and molecular cloning of the gene encoding the porcine phospholipase A<sub>2</sub> protein is described in detail previously by de Geus *et al.* (Nucl. Acid Res. 15, 3743-3759, 1987) and van den Bergh *et al.* (Eur. J. Biochem 170, 241-246, 1987).

[0047] With one of these well characterized clones, pCB08T, containing the entire PLA<sub>2</sub> cDNA sequence and *Kluyveromyces* specific genetic regulatory elements, we constructed the expression-cassette pKLAPLA-11 in order to obtain expression of porcine PLA<sub>2</sub> in the yeast *Kluyveromyces lactis*. As the PLA<sub>2</sub> cDNA sequence and the sequences for the *K.lactis* regulatory elements are all available in public databases, the skilled person can obtain all materials required to construct expression-cassettes for the expression of PLA<sub>2</sub> in *K.lactis*.

10 [0048] All standard molecular cloning techniques such as isolation and purification of nucleic acids, electrophoresis of nucleic acids, enzymatic modification, cleavage and/or amplification of nucleic acids, transformation of *E. coli*, etc., were performed as described in the literature (Sambrook *et al.* (1989) "Molecular Cloning: a laboratory manual", Cold Spring Harbour Laboratories, Cold Spring Harbour, New York; Innis *et al.* (eds.) (1990) and "PCR protocols, a guide to methods and applications" Academic Press, San Diego). Synthesis of oligo-deoxynucleotides and DNA sequence analysis were performed on Applied Biosystems 380B DNA synthesizer and 373A DNA sequencer, respectively, according to the user manuals supplied by the manufacturer.

20 [0049] To facilitate the construction of pKLAPLA-11 first appropriate flanking restriction sites were introduced at the borders of the mature PLAcDNA sequence by the Polymerase Chain Reaction (PCR). At the 5'-border, just at the cleavage site of the pro- and mature PLA<sub>2</sub> protein, a *Sma*I and at the 3'-border, just downstream the stop codon, *Xho*I and *Kpn*I restriction sites were introduced, simultaneously. To do so, two oligo nucleotides were synthesized:

25 Oligomer 1

mature PLA<sub>2</sub>

5' TGT CAT GCC CGG GCA TTA TGG CAG TTT CGT 3'

30 *Sma*I

35 Oligomer 2

5' AGT CCT CGG TAC CTC GAG TCA GCA GTA CTT CTT GGT GTC-3'

*Kpn*I

*Xho*I

stopcodon PLA<sub>2</sub>

40 [0050] The PCR amplification was performed with oligomers 1 and 2 as primers and pCB08T as template. The obtained amplified 400 bp PLAcDNA fragment was digested with *Sma*I and *Kpn*I and subsequently inserted into the appropriate sites of pTZ18R by molecular cloning. The resulting vector was designated pPLA-1. This modified PLAcDNA fragment was sequenced entirely to verify the PCR amplification reaction and the introduced flanking restriction sites.

45 [0051] To introduce a *Sa*I restriction site and optimal yeast translation initiation sequences at the 5'-border of the pre-signal sequence of PLA<sub>2</sub> two complementary synthetic oligo nucleotides were synthesized.

50 *Sa*I

SS<sub>pre</sub>

5' -TCG ACA AAA ATG AAA TTC CTC GTG TTG GCT GTT CTG CTC ACA-  
GT TTT TAC TTT AAG GAG CAC AAC CGA CAA GAC GAG TGT-

→ proPLA<sub>2</sub>

-GTG GGC GCT GCC CAG GAA GGC ATC AGC TCA A-3'  
 -CAC CCG CGA CGG GTC CTT CCG TAG TCG AGT T-5'

[0052] After annealing these two oligomers the obtained double-stranded DNA fragment was molecular cloned into the appropriate sites (*Sa*I and *Sma*I) of the pPLA-1. The obtained plasmid was designated pKLAPLA-5 and comprises the entire preproPLA<sub>2</sub>cDNA flanked at the 5'-end by a unique *Sa*I restriction site and at the 3'-end by an *Xho*I restriction site.

[0053] For expression of porcine PLA<sub>2</sub> in *K.lactis* the strong lactase promoter (P<sub>LAC</sub>) of *K.lactis* is used. Besides for the production of lactase in *K.lactis* this P<sub>LAC</sub> promoter sequence was used previously to express in *K.lactis* bovine chymosin (Berg van den J. *et al.*, 1990, *Bio/Technol.* 8, 135-139) and human serum albumin (HSA). For the expression of HSA plasmid pGBHSA-20 was constructed and described previously by Swinkels *et al.* 1993 (Antonie van Leeuwenhoek 64, 187-201).

[0054] For expression of HSA in *K.lactis* the HSAcDNA sequence in pGBHSA-20 is driven by the *K.lactis* LAC4 promoter. At the 3'-end the HSAcDNA is flanked by LAC4 terminator sequences. In addition, for selection of transformants, pGBHSA-20 contains the *Tn5* phosphotransferase gene which confers resistance to the antibiotic G418 (Geneticin, BRL; Reiss *et al.* (1984) *EMBO J.* 3, 3317-3322) driven by the *S.cerevisiae* ADH1 promoter (Bennetzen and Hall (1982) *J. Biol. Chem.* 257, 3018-3025). In the unique *Sst*II site of the LAC4 promoter pGBHSA-20 contains the *E. coli* vector pTZ19R which is used for amplification in *E.coli*. Prior to transformation of *K.lactis* the *E. coli* pTZ19R sequences are removed from pGBHSA-20 by *Sst*II digestion and agarose gel purification. Transformation of pGBHSA-20 linearized in the *Sst*II site of the LAC4 promoter into *K.lactis* results in integration into the genomic LAC4 promoter by homologous recombination.

[0055] For expression of porcine PLA<sub>2</sub> in *K.lactis* the preproPLA<sub>2</sub> sequence was fused appropriately to the *K.lactis* lactase promoter sequence in pGBHSA-20. Thereto pGBHSA-20 was digested with *Sa*I and *Xho*I and the HSAcDNA sequence was substituted for the *Sa*I-*Xho*I DNA fragment of pKLAPLA-5 by molecular cloning. As described above this *Sa*I-*Xho*I DNA fragment of pKLAPLA-5 comprises the preproPLA<sub>2</sub> encoding sequence. The final expression vector for PLA<sub>2</sub> was designated pKLAPLA-11.

[0056] Yeast transformants were generated by procedures as described in our published patent application EP-A 0 635 574, which are based on the method of Ito H. *et al.* (*J. Bacteriol.* 153, 163-168, 1983). pKLAPLA-11 was linearized in the LAC4 promoter by *Sst*II digestion. The pTZ19R sequences were removed by fractionation in and purification from agarose gels. 15 µg's of this DNA fragment were transferred into the *K.lactis* strains CBS 2360 and CBS 683 and G418-resistant colonies were obtained of both strains after incubation at 30°C for 3 days.

[0057] A limited number of transformants of each host strain were initially selected to test for expression of active porcine PLA<sub>2</sub> in the culture medium. Transformants were inoculated in *K.lactis* YEPD culture medium containing: 1% (w/v) yeast extract; 2% (w/v) peptone; 2% (w/v) glucose and 50µg/ml G418. After 3 days of growth at 30°C supernatants were collected and tested for the presence of active PLA<sub>2</sub> by the egg yolk activity assay as described below, after treatment of the samples with trypsin. Cleavage of the pro-peptide is necessary to activate the inactive pro-enzyme (produced by the *K.lactis* transformants) into active, mature PLA<sub>2</sub>.

[0058] All transformants appeared to produce active porcine PLA<sub>2</sub> ranging from 5 up to 40 U/ml.

[0059] One unit (IU) is defined as the amount of enzyme producing 1 micromole of free fatty acid per minute under standard conditions: egg yolk substrate (0.4% phospholipids), pH 8, 40°C, 6 mM Ca<sup>2+</sup>.

## Example 2

### Production of stable enzyme preparations

[0060] Broth of *Kluyveromyces lactis* is subjected to plate filtration followed by ultrafiltration. Ultrafiltrate is treated with 0.3% trypsin at pH 8.0 in the presence of 10 mM CaCl<sub>2</sub> during 2.5 hours which results in the removal of the heptapeptide of the pro-enzyme to activate the enzyme.

[0061] Benzoic acid and sorbic acid are added as a preservative at pH 4.0 and remaining trypsin activity was inactivated for 30 min. at 70°C. The final product is brownish and contains an activity of 10.000 IU/ml.

[0062] Stability of this preparation may be improved by further purification and storage at low temperatures. After one month of storage at 4°C no loss in enzyme activity is observed.

**Example 3****Application of phospholipase A2 in animal feed**

5 [0063] Trials are carried out with broilers to test the efficacy of phospholipase A2. Male broilers (Ross) are kept from day 1 to day 5 on a standard diet. At day 5, animals are selected from this group and are divided over cages. Weight of the animals and its variation are taken into account. The average weight and its deviation are equal per cage. Fifteen animals are kept in one cage. The cages are situated in an artificially heated, ventilated and illuminated broiler house. Floor space of each cage is 0.98 m<sup>2</sup>, with wire floors. The broiler house is illuminated for 24 hours per day. During the  
10 experimental period, light intensity is gradually reduced. The temperature is gradually reduced from 28°C during the first week to 23°C during the last week of the experiment. Humidity in the broiler unit is approximately 60% during the experimental period. Animals have been vaccinated against New Castle disease using the spray method at an age of respectively one and fourteen days. The experiment lasted 33 days comprising a pre-test period of 5 days and a test period of 28 days.

15 [0064] The experimental diets are offered *ad lib.* to the animals. Water is freely available.

[0065] Feed is cold pelleted (temperatures are kept below 65°C) at a diameter of 3mm.

The experiment comprises the following treatments:

- a) maize/wheat/soya diet (negative control)
- 20 b) maize/wheat/soya diet + 100 IU/kg
- c) maize/wheat/soya diet + 500 IU/kg

[0066] Each treatment is replicated six times (90 birds per treatment in total) Gain and feed conversion are measured. The composition of the feed used is shown in Table 1.

25

Table 1.

Composition of maize/wheat/soya diet in experiments with broilers.	
Ingredients	Contents (%)
Maize	25.0
Wheat	15.0
Soy oil	3.5
Animal fat	2.0
Manioc	11.68
35 Soy flour (50% crude protein)	19.45
Full fat toasted soy beans	10.0
Fishmeal	1.0
Meat meal tankage, high oil	4.0
40 Peas	5.0
Vitamins/mineral premix	1.0
Limestone	0.82
Monocalciumphosphate	1.00
Salt (NaCl)	0.30
45 DL-methionine	0.25
	100.00
ME broilers (MJ/kg)	12.55
Crude protein (%)	22.1
50 Crude fat (%)	9.6
Lysin (available) (%)	1.23 (1.04)
Methionine + Cysteine (available) (%)	0.91 (0.79)

Enzyme is added to this diet by mixing it first to a carrier.

55

[0067] The results are shown in Table 2.



Table 2.

Effect of phospholipase A2 in a maize/wheat/soya diet on growth and feed conversion ratio in broilers between 5 and 33 days of age.

	Feed intake g	Growth g	Feed conversions ratio
Basal diet	2613	1445	1.81
Diet + 100 IU/ kg feed	2569	1458	1.76
Diet + 500 IU/kg feed	2526	1472	1.72

[0068] An a second experiment, in essence identical to the one described above is performed in which a wheat/rye/soya diet as specified in Table 3 was used as basal diet.

The experiment comprises the following treatments:

- a) wheat/rye/soya diet (negative control)
- b) wheat/rye/soya diet + 100 IU/kg
- c) wheat/rye/soya diet + 500 IU/kg
- d) wheat/rye/soya diet + 1000 IU/kg

All other parameters are as described above for the maize/wheat/soya experiment.

Table 3.

Composition of Wheat/rye/soya diet in experiments with broilers.

Ingredients	Contents (%)
Wheat	40.0
Rye	10.0
Soy oil	1.0
Animal fat	6.0
Manioc	4.28
Soya bean meal (45.4 % crude protein)	22.0
Full fat toasted soy beans	10.0
Meat meal tankage (58 crude protein)	3.0
Vitamins/mineral premix	1.0
Limestone	0.94
Monocalciumphosphate	1.20
Salt (NaCl)	0.26
L-lysine HCl	0.11
DL-methionine	0.21
	100.00
ME broilers (MJ/kg)	11.9
Crude protein (%)	21.4
Crude fat (%)	10.5
Lysin (available) (%)	1.23 (1.05)
Methionine + Cysteine (available) (%)	0.90 (0.77)

Enzyme is added to this diet by mixing it first to a carrier.

[0069] The results are shown in Table 4.

Table 4.

Effect of phospholipase A2 in a wheat/rye/soya diet on growth and feed conversion ratio in broilers between 5 and 33 days of age.

	Feed intake g	Growth g	Feed conversions ratio
Basal diet	2752	1556	1.77
Diet + 100 IU/kg feed	2747	1568	1.75
Diet + 500 IU/kg feed	2733	1586	1.72
Diet + 1000 IU/kg feed	2724	1572	1.73

[0070] As is shown in Table 4, there is an optimum in the range of phospholipase concentrations to be used in this particular broiler diet, i.e. more than about 100 IU/kg feed and less than about 1000 IU/kg of feed. For other systems different optima may exist which can be determined by routine experiments.

#### Example 4

#### Application of phospholipase A2 in milk replacers

[0071] An experiment is performed using 3 groups of each 5 male Friesian Dutch Holstein- Friesian calves.

[0072] During the pre-experimental period a commercial milk replacer is fed. After 14 days, animals are divided to receive three treatments, taking weight and variations in weight into account. The animals are kept in individual boxes. The stable is lighted naturally; it is ventilated and is kept at a temperature of about 18°C.

[0073] The animals are adapted to their diet during 14 days. Subsequently, faeces is collected quantitatively for 5 consecutive days for 24 hours per day. Calves are harnessed before the experimental period. Faeces is collected in plastic bags attached to the harness. Once a day, faeces is weighed, pooled and stored at -20°C. Prior to analyses, faeces is thoroughly mixed and subsampled.

[0074] Animals are fed individually according to their weight following a feeding scheme. The milk replacer used has the following composition:

	%
Skim milk powder	58.5
Fat	19.8
Lactose	17.6
Starch, vitamins, minerals	4.1
ME	4450 kcal/kg
Crude protein (N*6.25)	21.5%
Crude fat	19.5%

[0075] Milk replacer powder is mixed with water before feeding and fed at a temperature of about 40°C.

[0076] According to the treatment, the fat consists of 18% beef tallow, coconut fat or lard. Lecithin is added at a concentration of 10% of fat content.

[0077] Porcine phospholipase A2 is added to these diets at a final concentration of 500 IU/kg of milk replacer. Digestibility of fat is measured. Results are shown in Table 5.

Table 5.

The effects of phospholipase A2 treatment on the digestibility of various fats by non-ruminating calves receiving 18% fat in their diets and 1.8% lecithin (10% of fat content). Phospholipase A2 is prepared as shown in example 2 and is added to the diet at a final concentration of 500 IU/kg of milk replacer.

	Without phospholipase A2	With phospholipase A2
Beef tallow	70.0%	73.1 %
Coconut fat	95.6%	96.2%

Table 5. (continued)

	Without phospholipase A2	With phospholipase A2
Lard	79.4%	84.3%

## SEQUENCE LISTING

[0078]

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Gist-brocades B.V.  
 (B) STREET: Wateringseweg 1  
 (C) CITY: Delft  
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(ii) TITLE OF INVENTION: Application of phospholipases in animal feed

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTCATGCCC GGGCATTATG GCAGTTTCGT

30

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs  
(E) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

10

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15

AGTCCTCGGT ACCTCGAGTC AGCAGTACTT CTTGGTGTC

39

(2) INFORMATION FOR SEQ ID NO: 3:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 73 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

35

TCGACAAAAA TGAAATTCCT CGTGTTGGCT GTTCTGCTCA CAGTGGGCGC TGCCCAGGAA  
GGCATCAGCT CAA

73

40

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: DNA (genomic)

50

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGAGCTGAT GCCTTCCTGG GCAGCGCCCA CTGTGAGCAG AACAGCCAAC ACGAGGAATT  
TCATTTTGG

69

5

# Claims

- 10 1. A process for improving the efficiency of feed utilization in which an animal is fed a diet which comprises a composition obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance.
2. A process for promoting the growth of animals in which an animal is fed a diet which comprises a composition obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance.
- 15 3. An animal feed composition improving the efficiency of feed utilization, obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance.
4. A growth-promoting animal feed composition obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance.
- 20 5. A composition according to claim 3 or 4, which comprises phospholipid.
6. A composition according to claim 5, wherein the phospholipid comprises lecithin.
- 25 7. A composition according to any one of claims 3 to 6, in which the phospholipase is obtainable from a mammal, a plant or a microorganism.
8. A composition according to claim 7, wherein the phospholipase is a mammalian phospholipase A2 selected from the group comprising bovine, porcine, murine, rat and human phospholipase A2.
- 30 9. A composition according to any one of claims 3 to 8 in which the phospholipase is obtainable by expression of recombinant DNA in a host organism.
10. A composition according to claim 9 in which the host organism is a microorganism selected from the group comprising bacteria, yeasts and filamentous fungi.
- 35 11. A composition according to claim 10 in which the microorganism is of a genus selected from *Bacillus*, *Escherichia*, *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, *Yarrowia*, *Candida*, *Aspergillus*, *Trichoderma*, *Penicillium*, *Mucor*, *Fusarium* and *Humicola*.
- 40 12. A composition according to claim 11 in which the microorganism is *Escherichia coli*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Aspergillus niger*.
13. A composition according to any one of claims 3 to 9, which comprises plant material containing the phospholipase obtainable from a transgenic plant.
- 45 14. A composition according to claim 13, which comprises the seeds of a transgenic plant containing phospholipase obtainable by expression of recombinant DNA.
- 50 15. A composition according to any one of claims 5 to 14, which comprises from 1,000 to 5,000,000 International Units of phospholipase per kg of phospholipid.
16. A composition according to any one of claims 3 to 15, wherein the phospholipase additive is present at a concentration in the range of 100 to 1000 International Units per kg of feed.
- 55 17. A process for the production of an animal feed according to any one of claims 3 to 16 which comprises mixing a ready for use phospholipase with feed substance.

18. A process according to claim 17, which comprises mixing a ready for use phospholipase with feed substance and at least one phospholipid.
19. A process according to claims 17 or 18, wherein the phospholipase is produced recombinantly.
20. A process according to claim 19, wherein the phospholipase is added in the form of transgenic plant material.
21. A process according to claim 20, wherein the transgenic plant material is processed so as to make available the phospholipase.
22. Use of a composition obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance, to feed non-ruminating animals.
23. Use of a composition obtainable by a process comprising mixing a ready for use phospholipase additive with feed substance, to feed non-ruminating calves.

#### Patentansprüche

1. Verfahren zum Verbessern der Wirksamkeit einer Futtermittelverwendung, bei der ein Tier mit einer Diät gefüttert wird, welche eine Zusammensetzung enthält, die durch ein Verfahren erhältlich ist, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff beinhaltet.
2. Verfahren zum Fördern des Wachstums von Tieren, bei dem ein Tier mit einer Diät gefüttert wird, welche eine Zusammensetzung enthält, die durch ein Verfahren erhältlich ist, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff umfasst.
3. Tierfutterzusammensetzung, welche die Wirksamkeit der Futtermittelverwendung verbessert, erhältlich durch ein Verfahren, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff umfasst.
4. Wachstumsfördernde Tierfutterzusammensetzung, erhältlich durch ein Verfahren, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff umfasst.
5. Zusammensetzung nach Anspruch 3 oder 4, die ein Phospholipid enthält.
6. Zusammensetzung nach Anspruch 5, worin das Phospholipid Lecithin enthält.
7. Zusammensetzung nach einem der Ansprüche 3 bis 6, bei der die Phospholipase aus einem Säugetier, einer Pflanze oder einem Mikroorganismus erhältlich ist.
8. Zusammensetzung nach Anspruch 7, worin die Phospholipase eine Säugetierphospholipase A2 ist, ausgewählt aus Rinder-, Schweine-, Mäuse-, Ratten- und Humanphospholipase A2.
9. Zusammensetzung nach einem der Ansprüche 3 bis 8, bei der die Phospholipase durch Expression einer rekombinanten DNA in einem Wirtsorganismus erhältlich ist.
10. Zusammensetzung nach Anspruch 9, bei welcher der Wirtsorganismus ein Mikroorganismus ist, ausgewählt aus Bakterien, Hefen und Fadenpilzen.
11. Zusammensetzung nach Anspruch 10, bei welcher der Mikroorganismus aus den Gattungen Bacillus, Escherichia, Saccharomyces, Kluyveromyces, Hansenula, Pichia, Yarrowia, Candida, Aspergillus, Trichoderma, Penicillium, Mucor, Fusarium und Humicola ausgewählt ist.
12. Zusammensetzung nach Anspruch 11, bei welcher der Mikroorganismus Escherichia coli, Saccharomyces cerevisiae, Kluyveromyces lactis oder Aspergillus niger ist.
13. Zusammensetzung nach einem der Ansprüche 3 bis 9, die ein Pflanzenmaterial aufweist, das die Phospholipase

enthält, die aus einer transgenen Pflanze erhältlich ist.

- 5 14. Zusammensetzung nach Anspruch 13, welche die Samen einer transgenen Pflanze aufweist, die eine Phospholipase enthält, welche durch Expression einer rekombinanten DNA erhältlich ist.
15. Zusammensetzung nach einem der Ansprüche 5 bis 14, die 1.000 bis 5.000.000 Internationale Einheiten von Phospholipase pro kg Phospholipid enthält.
- 10 16. Zusammensetzung nach einem der Ansprüche 3 bis 15, worin das Phospholipase-Additiv mit einer Konzentration im Bereich von 100 bis 1.000 Internationalen Einheiten pro kg Futtermittel vorliegt.
17. Verfahren zur Herstellung eines Tierfuttermittels nach einem der Ansprüche 3 bis 16, welches das Mischen einer gebrauchsfertigen Phospholipase mit einem Futtermittelstoff umfasst.
- 15 18. Verfahren nach Anspruch 17, welches das Mischen einer gebrauchsfertigen Phospholipase mit einem Futtermittelstoff und mindestens einem Phospholipid umfasst.
19. Verfahren nach Anspruch 17 oder 18, worin die Phospholipase rekombinant gebildet worden ist.
- 20 20. Verfahren nach Anspruch 19, worin die Phospholipase in Form eines transgenen Pflanzenmaterials zugegeben wird.
21. Verfahren nach Anspruch 20, worin das transgene Pflanzenmaterial derart verarbeitet wird, daß die Phospholipase zugänglich gemacht wird.
- 25 22. Verwendung einer Zusammensetzung, die durch ein Verfahren erhältlich ist, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff umfasst, zum Füttern von nichtwiederkäuenden Tieren.
- 30 23. Verwendung einer Zusammensetzung, die durch ein Verfahren erhältlich ist, welches das Mischen eines gebrauchsfertigen Phospholipase-Additivs mit einem Futtermittelstoff umfasst, zum Füttern von Nichtwiederkäuenden Kälbern.

### 35 Revendications

- 40 1. Procédé pour améliorer le rendement de l'utilisation de la nourriture, dans lequel un animal est nourri d'un régime qui comprend une composition susceptible d'être obtenu par un procédé comprenant le mélange à la substance nutritive d'un additif phospholipase prêt à l'emploi.
2. Procédé pour favoriser la croissance d'animaux, dans lequel un animal est nourri d'un régime qui comprend une composition susceptible d'être obtenu par un procédé comprenant le mélange à la substance nutritive d'un additif phospholipase prêt à l'emploi.
- 45 3. Composition pour l'alimentation des animaux améliorant le rendement de l'utilisation de la nourriture, susceptible d'être obtenu par un procédé comprenant le mélange à la substance nutritive d'un additif phospholipase prêt à l'emploi.
- 50 4. Composition pour l'alimentation des animaux favorisant leur croissance, susceptible d'être obtenu par un procédé comprenant le mélange à la substance nutritive d'un additif phospholipase prêt à l'emploi.
5. Composition selon la revendication 3 ou 4, qui comprend un phospholipide.
6. Composition selon la revendication 5, dans laquelle le phospholipide comprend de la lécithine.
- 55 7. composition selon l'une quelconque des revendications 3 à 6, dans laquelle la phospholipase est susceptible d'être obtenue à partir d'un mammifère, d'une plante ou d'un micro-organisme.

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8. Composition selon la revendication 7, dans laquelle la phospholipase est une phospholipase de mammifère A2, choisie dans le groupe comprenant la phospholipase A2 bovine, porcine, de souris, de rat et humaine.
- 5 9. Composition selon l'une quelconque des revendications 3 à 8, dans laquelle la phospholipase est susceptible d'être obtenue par expression d'ADN recombinant dans un organisme hôte.
10. Composition selon la revendication 9, dans laquelle l'organisme hôte est un micro-organisme choisi dans le groupe comprenant les bactéries, les levures et les champignons filamenteux.
- 10 11. Composition selon la revendication 10, dans laquelle le micro-organisme est d'un genre choisi parmi *Bacillus*, *Escherichia*, *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, *Yarrowia*, *Candida*, *Aspergillus*, *Trichoderma*, *Penicillium*, *Mucor*, *Fusarium* et *Humicola*.
12. Composition selon la revendication 11, dans laquelle le micro-organisme est *Escherichia coli*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis* ou *Aspergillus niger*.
- 15 13. Composition selon l'une quelconque des revendications 3 à 9, qui comprend de la matière végétale contenant la phospholipase susceptible d'être obtenu d'une plante transgénique.
- 20 14. Composition selon la revendication 13, qui comprend les semences d'une plante transgénique contenant de la phospholipase, susceptible d'être obtenu par expression d'ADN recombinant.
15. Composition selon l'une quelconque des revendications 5 à 14, qui comprend de 1.000 à 5.000.000 unités internationales de phospholipase par kg de phospholipide.
- 25 16. Composition selon l'une quelconque des revendications 3 à 15, dans laquelle l'additif de phospholipase est présent en une concentration dans la gamme de 100 à 1000 Unités internationales par kg de substance nutritive.
- 30 17. Procédé de production d'une nourriture pour animaux selon l'une quelconque des revendications 3 à 16, qui comprend l'étape consistant à mélanger une phospholipase prête à l'emploi à une substance nutritive.
18. Procédé selon la revendication 17, qui comprend l'étape consistant à mélanger une phospholipase prête à l'emploi à une substance nutritive et au moins un phospholipide.
- 35 19. Procédé selon les revendications 17 ou 18, dans lequel la phospholipase est produite par recombinaison.
20. Procédé selon la revendication 19, dans lequel la phospholipase est additionnée sous forme de matière végétale transgénique.
- 40 21. Procédé selon la revendication 20, dans lequel la matière végétale transgénique est traitée pour obtenir la phospholipase.
22. Utilisation d'une composition susceptible d'être obtenu par un procédé qui comprend l'étape consistant à mélanger un additif phospholipase prêt à l'emploi à une substance nutritive, pour nourrir des animaux non ruminants.
- 45 23. Utilisation d'une composition susceptible d'être obtenu par un procédé qui comprend l'étape consistant à mélanger un additif phospholipase prêt à l'emploi à une substance nutritive, pour nourrir des veaux non ruminants.
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- 55